DOI 10.5012/bkcs.2011.32.5.1500

Non-Essential Activation of Co^{2+} and Zn^{2+} on Mushroom Tyrosinase: Kinetic and Structural Stability

N. Gheibi,[*] A. A. Saboury,[†] and M. Sarreshtehdari[‡]

Cellular and Molecular Research Center and Department of Biophysics, Qazvin University of Medical Sciences, Qazvin, Iran
[*]E-mail: gheibi_n@yahoo.com
[†]Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran
[‡]Department of Internal Medicine, School of Medicine, Qazvin University of Medical Sciences, Qazvin, Iran
Received November 8, 2010, Accepted March 3, 2011

Tyrosinase is a widespread enzyme with great promising capabilities. The Lineweaver-Burk plots of the catecholase reactions showed that the kinetics of mushroom tyrosinase (MT), activated by Co^{2+} and Zn^{2+} at different pHs (6, 7, 8 and 9) obeyed the non-essential activation mode. The binding of metal ions to the enzyme increases the maximum velocity of the enzyme due to an increase in the enzyme catalytic constant (k_{cat}). From the kinetic analysis, dissociation constants of the activator from the enzyme-metal ion complex (K_a) were obtained as $5 \times 10^4\ M^{-1}$ and $8.33 \times 10^3\ M^{-1}$ for Co^{2+} and Zn^{2+} at pH 9 and 6 respectively. The structural analysis of MT through circular dichroism (CD) and intensive fluorescence spectra revealed that the conformational stability of the enzyme in these pHs reaches its maximum value in the presence of each of the two metal ions.

Key Words : Mushroom tyrosinase, Non-essential activation, Co^{2+}, Zn^{2+}, pH

Introduction

The presence of tyrosinase (EC 1.14.18.1), the key enzyme for the synthesis of melanins, is reported in a variety of tissues and within different stages of lifespan. This enzyme, as a ubiquitous cuproenzyme, utilizes phenolic compounds and transforms these substances into the corresponding *o*-quinones.[1] The polymerization of *o*-quinones results in the formation of melanin which is an extremely vital process from pathophysiological point of view.[2,3] Furthermore, recent studies have confirmed the contribution of tyrosinase to quite diverse natural processes.[4-6] Tyrosinase family contains two highly conserved putative copper-binding catalytic domains which are highly homologous with the copper-binding catalytic domains of bacterial, fungal and mammalian tyrosinase.[7] Tyrosinases, in particular the edible mushroom tyrosinase (MT), have been focus of different mechanistic studies due to their availability and close similarity with mammals' tyrosinase.[8] So far, several bivalent cations have been found to be inhibitors or activators of tyrosinase, a point that has not reveived due consideration. One reason for this discrepancy might be the purity of the enzyme preparations (crude and highly purified enzyme), while the others may be associated with the source of the enzyme. The impacts of Fe^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, or Cd^{2+} as divalent metal ions on kinetics of Sepia officinalis tyrosinase activity have been studied.[9] DOPA-oxidase activity of tyrosinase extracted from liver pigment cells of Rana Esculenta L. has been reported to increase in a concentration dependent manner in the presence of copper. The activation was attributed to the transfer of the copper ions to the apoenzyme.[10] In spite of some weak binding sites on MT for Co^{2+} or Zn^{2+}, these cations produce no effect on the catalytic activities of the native or reconstituted metapotyrosinase.[11]

It is possible that some of the activation phenomena observed were due to the activation by metals or the reducing agents. As mentioned above, similarities between the MT and other tyrosinases, in particular the mammal tyrosinase, have made it an apt example for *in-vitro* studies in this field.[8,12] Our previous study on MT kinetics revealed that the cresolase activity on *p*-coumaric acid was boosted in the presence of Cu^{2+} and Ni^{2+} although inhibited when phenol, L-tyrosine, or 4-[(4-methylphenyl)azo]-phenol were used as substrates. Similarly, catecholase activity on caffeic acid was enhanced in the presence of the two ions, but inhibited when catechol, L-DOPA, or 4-[(4-methylbenzo)-azo]-1,2-benzenediol were applied as substrates.[13] Thus, consistent with our previous studies and many other numerous reports on the stability, kinetics, and inhibition of both activities of MT,[14-17] the present comprehensive kinetic study on catecholase activity of MT elucidates the effect of Co^{2+} and Zn^{2+} on the enzyme kinetics and structure. Yet, some important aspects of the enzyme structure, mechanism, and behavior have remained unresolved. Considering the importance of the enzyme-divalent metal ion interactions,[18-22] the regulative and catalytic roles of Co^{2+} and Zn^{2+} in melanogenesis pathway,[23-25] and the fact that melanosomal pH is an essential factor which regulates multiple stages of melanin structures,[26] this study was devoted to further explore the effects of Co^{2+} and Zn^{2+} on MT kinetics and structure through circular dichroism (CD) and spectroflourimetric techniques at different pHs.

Experimental

Materials. Mushroom tyrosinase (MT; EC 1.14.18.1),

specific activity 3400 units/mg, was purchased from Sigma. 3,4-dihydroxycinnamic acid (caffeic acid, λ_{max} = 310 nm, ε = 12000 $M^{-1}.cm^{-1}$) and analytical grade of $CoSO_4$ and $ZnSO_4$ from Merck™. The salts of 2.5 mM phosphate buffer (PBS) were purchased from Merck. All experiments were carried out at 20 °C and solutions were prepared in de-ionized distilled water.

Methods.

Kinetic Measurements: The kinetic assays of catecholase activity were carried out using Cary spectrophotometer, 100 Bio model, with jacketed cell holders. Caffeic acid as diphenol substrate was used in phosphate buffer (2.5 mM) at pH 6.8 in a conventional quartz cell thermostated to maintain the temperature at 20 ± 0.1 °C for 2 min at an enzyme concentration of 11.8 μM (40 unit/mL). To study the effect of pH, 2.5 mM PBS buffers at different pHs of 6, 7, 8 and 9 were used. Kinetic measurements of catecholase reaction with caffeic acid at different pHs were carried out both in the absence and presence of Co^{2+} and Zn^{2+} (0.2, 0.6 and 0.9 mM). All assays were repeated as triplicate. Units were defined by the vender. Accordingly, one unit of the enzyme in the cresolase activity is equal to the 0.001 change in the optical density of L-tyrosine per minute at 280 nm in 3 mL of the reaction mixture at 25 °C and pH 6.5.

Circular Dichroism Spectroscopy: The far UV region (190-260) corresponding to the peptide bond absorption was analyzed by an Aviv model 215 Spectropolarimeter (Lakewood, USA) to obtain the content of regular secondary structure in MT. For the far UV spectra, MT was studied at a concentration of 0.21 mg/mL using 1 mm path length quartz cell. Protein solutions were prepared using 2.5 mM PBS buffer at pH 6.8. The protein solutions were also incubated with Co^{2+} (0.2 mM) and Zn^{2+} (0.2 mM) for at least 5 min. Then the experiments for sole MT and its incubation with the metal ions were carried out at pH range of 6, 7, 8 and 9. The results were expressed as ellipticity [deg·cm^2·$dmol^{-1}$)] based on a mean amino acid residue weight (MRW) of 125 for MT having the average molecular weight of 120 kD. The molar ellipticity was determined as [θ] = (100 × (MRW) × θ_{obs}/CL), where θ_{obs} is the observed ellipticity in degrees at a given wavelength, C is the protein concentration in mg/ml and L is the length of the light path in cm. All measurements were carried out at 25 °C with the help of a thermostatically controlled cell holder attached to a Neslab's RTE-110 circulating water bath with an accuracy of ± 0.1 °C.

Fluorescence Spectroscopy: Intrinsic fluorescence spectra of MT were recorded with Cary spectrofluorimeter model Bio100 with excitation wavelength of 280 nm and the tryptophan residues emission spectra were recorded from 300 to 400 nm. Intrinsic fluorescence of sole enzyme (0.17 mg/mL), and its incubation by 0.2 mM Co^{2+} and Zn^{2+} for 5min in pH range of 6, 7, 8 and 9 were measured in 2.5 mM PBS buffer at pH 6.8 and 25 °C, in 3mL quartz cuvettes with a 1-cm excitation light path.

Results and Discussion

MT Activation by Co^{2+} and Zn^{2+} in the Catecholase

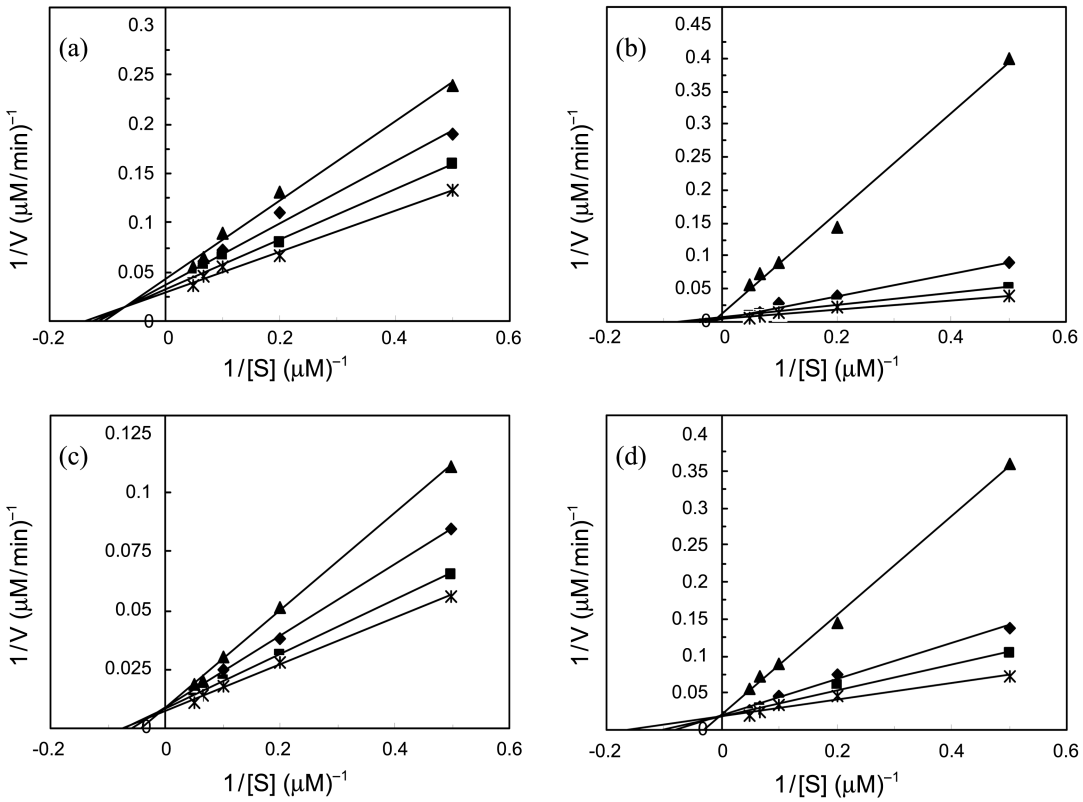


Figure 1. MT kinetic assays for catecholase reactions of caffeic acid in 2.5 mM phosphate buffer at pHs = 6(a), 7(b), 8(c) and 9(d) without (▲) and in the presence of different fixed concentrations of Co^{2+}: 0.2 mM (◆), 0.6 mM (■) and 0.9 mM (∗).

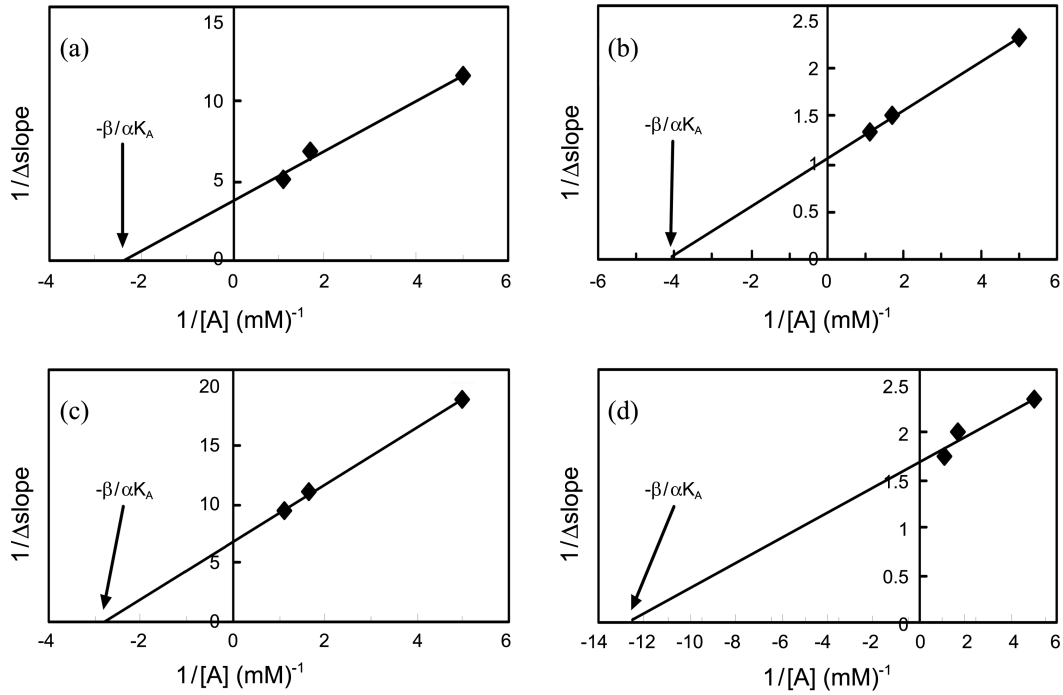
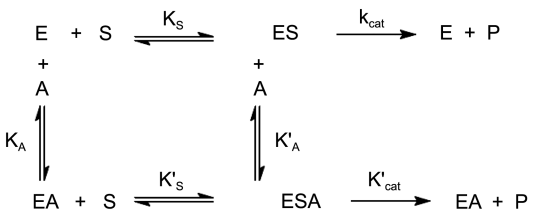


Figure 2. The secondary plot, 1/Δ slope against 1/ΔA(Co^{2+}) at pHs = 6(a), 7(b), 8(c) and 9(d).

Reactions. Catecholase reactions were examined at pH 6, 7, 8 and 9 at 20 °C and three concentrations 0.2, 0.6 and 0.9 mM of metal ions. Data collection and analysis of MT kinetic reactions with the metal ions in catecholase reactions showed a special case of non-essential activation. The reciprocal plot for the non-essential activation at pH points mentioned earlier are illustrated in two series of figures, i.e. Figures 1 and 2 for Co^{2+} and Zn^{2+}, respectively. The figures show a series of straight lines intersecting each other on the left hand side of the vertical axis, over the horizontal axis, indicating the activation of MT at low concentrations of metal ions. The apparent maximum velocity (V'_{max}) and the apparent dissociation constant of the substrate (K'_S) values, can be obtained at any fixed concentration of metal ions from the vertical (Y) and abscissa (X) intercept, respectively. As illustrated in Scheme 1, the non-essential activation of MT by metal ion as an activator molecule (A) is proposed by rapid equilibrium model. In this model, $V'_{max} = V_{max}$ (1 + β[A]/αK_A)/(1 + A]/αK_A) and K'_S = KS(1 + [A]/αK_A)/(1 + [A]/αK_A).[27] K_S is the dissociation constant of the substrate from the enzyme, K_A is the dissociation constant of the effector from the enzyme, α and β represent maximal changes in K_S and V_{max} of the enzyme in the presence of different concentrations of metal ions as activators [A]. V_{max} and K_S values are obtained from the Y-intercept and X-intercept of reciprocal plots, respectively. The magnitudes of slope and Y-intercept from the reciprocal plots were obtained and inversely replotted *vs.* inverse concentration of the activator in series of figures marked as Figures 3 and 4 for Co^{2+} and Zn^{2+}, respectively. The linear plot of 1/Δ slope against 1/ΔA revealed the Y-intercept of βVmax/(β-1) and the X-intercept of -β/αK_A. The linear plot of 1/ΔY-intercept against 1/ΔA shows the Y-intercept of $\beta V_{max}/K_S$ (β-α) and the X-intercept of -β/αK_A. The α, β, K_S and K_A values were calculated from these secondary plots and are illustrated in Table 1. The α values (α < 1) obtained herein suggest that the binding of metal ions to the enzyme increases the binding affinity of the substrate. The β values (β > 1) obtained likewise suggest that the binding of metal ion to the enzyme increases the maximum velocity of the enzyme due to an increase in the enzyme catalytic constant (k_{cat}). The results show that Co^{2+} and Zn^{2+} activate MT through a concentration dependent manner. The affinity of binding for the metal ion (as an activator) is greater than the affinity for its role as an inhibitor ($K_A < K_S$).



Scheme 1. Non essential activation model of MT activation by Co^{2+} or Zn^{2+} (A).

The α parameter determines the activation effect at low [S] and the β determines the activation effect at high [S]. When α < 1 or β > 1, there is "positive" activation towards the reaction; and when α > 1 or β < 1, there is "negative" activation towards the reaction. As presented in Table 1, magnitudes of the activator affinity to enzyme (K_a) are several times greater than the substrate affinity to the MT ($1/K_s$).

Structural Analysis of MT in the Presence of Co^{2+} and Zn^{2+} at Different pHs. The analysis of the secondary

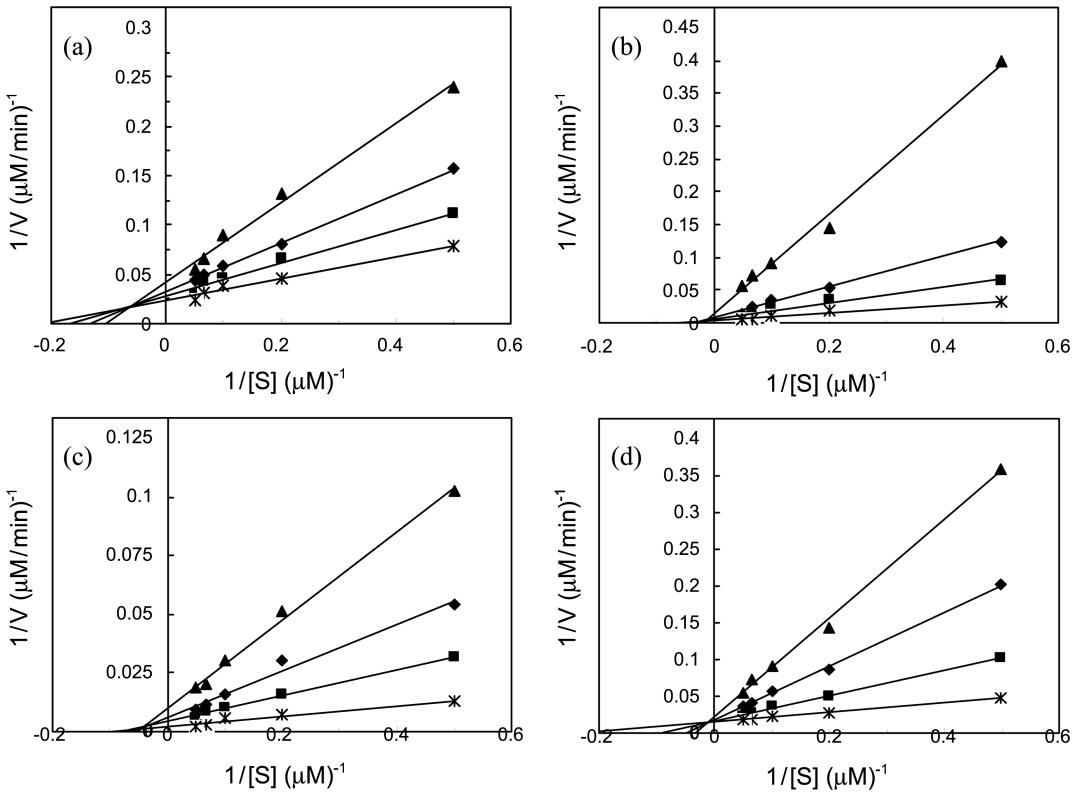


Figure 3. MT kinetic assays for catecholase reactions of caffeic acid in 2.5 mM phosphate buffer at pHs = 6(a), 7(b), 8(c) and 9(d) without (▲) and in the presence of different fixed concentrations of Zn^{2+}: 0.2 mM (◆), 0.6 mM (■) and 0.9 mM (∗).

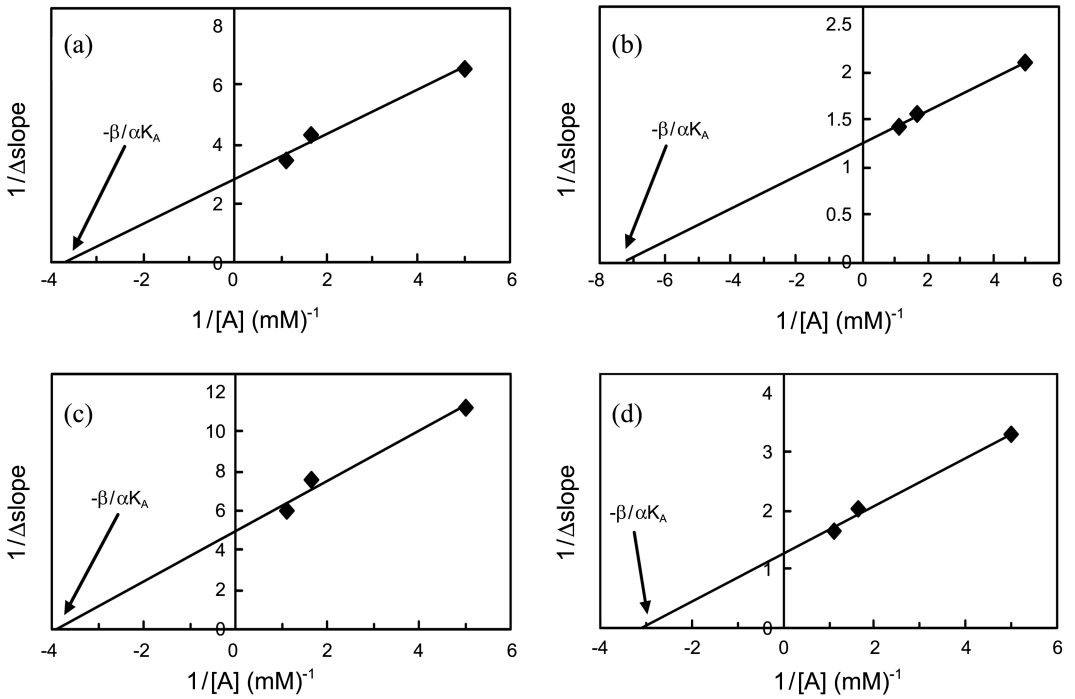


Figure 4. The secondary plot, 1/Δ slope against $1/\Delta A(Zn^{2+})$ at pHs = 6(a), 7(b), 8(c) and 9(d).

structure of MT from CD spectra was carried out using CD spectra deconvlusion software the results of which are presented in Table 2. The results rule out a significant change in the secondary structure of MT in the presence of metal ions at different pHs. Figure 3 supports the observable changes in the tertiary structure of the sole enzyme and the enzyme incubated with Co^{2+} and Zn^{2+} at different pHs. Conformational changes of an enzyme revealed a decrease

Table 1. Kinetic parameters obtained from non-essential activation model in the presence and absence of 0.2 mM Co^{2+} and Zn^{2+} at different pHs

	Co^{2+}				Zn^{2+}				Enzyme	
pH	α	β	K_a $(M)^{-1}$	K_A (mM)	α	β	K_a $(M)^{-1}$	K_A (mM)	V_{max} (mM/min)	K_s (mM)
6	0.42	3.04	1.35×10^3	0.74	0.24	2.01	8.33×10^3	0.12	23.3	9.3
7	−0.9	3.64	-1.33×10^3	−0.75	−0.18	3.62	-1.11×10^4	−0.09	79.4	60.4
8	0.23	1.05	1.43×10^4	0.07	−1.1	15.9	-0.22×10^3	−4.6	99	18.6
9	0.15	1.23	5×10^4	0.02	−0.29	1.53	-1.1×10^4	−0.12	46.7	31.3

in the intrinsic fluorescence spectra of MT. The plot of fluorescence spectra in the figure shows that the maximum intensity in 350 nm decreased at pH 8 for the sole enzyme and the MT incubated with Co^{2+} and Zn^{2+} at pHs 9 and 6, respectively . It has been shown that the maximum fluorescence spectra (350 nm) of MT incubated with Zn^{2+} (0-50 mM) using an excitation wavelength of 280 nm decreased with a slight red shift in a dose dependent manner.[28] Similar results were achieved for the high concentration of NaCl (more than 2.0 M) with the maximum fluorescence spectra in 350 nm using an excitation wavelength of 280 nm.[29] The lowest level of fluorescence intensity in the presence of Co^{2+} and Zn^{2+} was obtained at pH 9 and pH 6, respectively. Beltramini *et al*.[30] have reported that upon excitation of apo-, met-, and oxy-tyrosinase at 294 nm, where 94% of light was absorbed by tryptophan residues, tyrosinase exhibited fluorescence with a maximum intensity near 330 nm. Besides, it has been reported that there are differences between the spectra obtained under excitation at 275 and 294 nm.[31] The enzyme used in our study was a mixed type of met-and oxy-tyrosinase and with the excitation wavelength of 280 nm it showed a maximum intensity in 350 nm.

The CD spectra of MT in Figure 6 represent two negative peaks at 208 and 222 nm. These two points are ascribed to the α-helix and β-sheet structures. The negative broad peaks at 208 and 222 nm were analyzed by CD deconvlusion software (CDNN version 2.1). As presented in Table 2, the content of regular secondary structures (59.2%) for Co^{2+} and (59.8%) for Zn^{2+} revealed enzyme stability after its incubation with 0.2 mM concentration of these cations at pHs 9 and 6, respectively. It showed that the percent of β-sheet structures of MT increased significantly for the sole enzyme at pH 7 to the highest values after being incubated with Co^{2+} and Zn^{2+} at pHs 9 and 6, respectively, while the α-helix structures slightly decreased in these points. The explanation is that a subtle re-arrangement of the MT structure occurred in the mentioned pH condition.

As presented in Table 1, the higher K_a values for Co^{2+} and Zn^{2+} at pHs 9 and 6 show a good concurrence with the compactness and thus higher secondary and tertiary structural stability of MT at the mentioned pHs. Considering the results of the present study, the difference in metal affinity to the enzyme at different pHs reveals the diverse impact of Co^{2+} and Zn^{2+}, leading to a change in the enzyme stability. The other important finding of this study is the high binding affinity for metal ions. Binding constants in the milimolar range would lead to the conclusion that such interactions are physiologically relevant. There are several studies regarding the physiological effect of transition metal ions on the melanogenesis process. These studies have mainly focused on nonenzymatic aspects of melanins such as cation exchange properties, structural modification by metal ions and rearrangement of dopachrome.[23,24] However, in the melanin-containing tissues there are high qualities of some heavy metals, in particular zinc, copper, and iron,[32] for example, high levels of such metal ions have been found in the choroids of eye,[33,34] black hair,[35] pigmented moles,[36] and human melanomas.[37] The binding of transition metals to the MT leads to the structural and functional changes as a consequence of such interactions. In our previous study, the Cu^{2+} and Ni^{2+} interactions with MT activity were shown to cause some conformational changes, which made the enzyme more fragile. They behaved similarly in the catalytic activities and boosted MT activity, but at varying degrees and at pH 6.8.[13] It is proposed that the divalent metal ions, more or less, get in the way of the interaction between the substrate and the MT active site *via* a coordination binding. This indicates that both the presence and binding of the caffeic acid carboxyl group with metal ions confer its interaction with MT active site.

The halide ions such as F^{-1} and Cl^{-1} inhibit tyrosinase through direct interaction with the active site.[38,39] In the

Table 2. The percent of secondary structure calculated from deconvlusion of UV-CD spectra for sole enzyme and in the presence of 0.2 mM Co^{2+} and Zn^{2+} at different pHs

	Enzyme				Co^{2+}				Zn^{2+}			
	pH											
Structure	6	7	8	9	6	7	8	9	6	7	8	9
α-Helix	21.1	20.7	20.1	18.7	21.5	20.1	19.8	17.2	17.1	19.4	18.7	21.1
β-Sheet	35.3	34.8	34.9	40.3	34.2	34.9	37.5	42	42.7	38.3	35.7	35.3
β-Turn	16.7	15.9	15.9	15.1	16.6	15.9	15.9	13.6	14	15.7	13.1	16.7
Random Coil	26.9	28.6	29.1	25.9	26.5	28.1	26.8	27.2	26.2	26.6	32.5	26.9

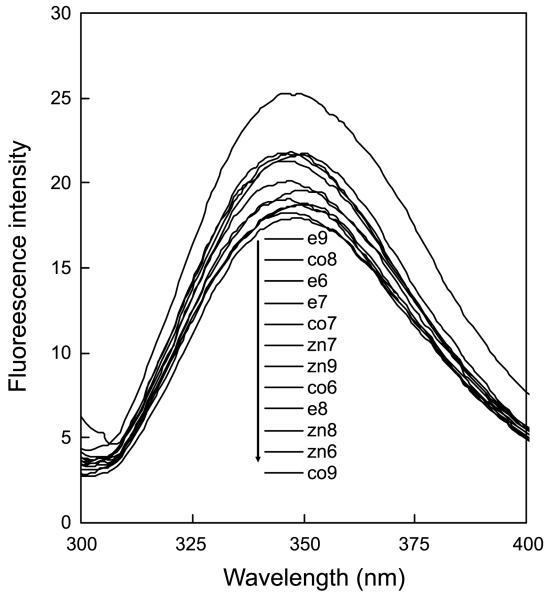


Figure 5. Intrinsic fluorescence emission spectra of MT without (e) and with 0.2 mM Co^{2+} and Zn^{2+} in 2.5 mM PBS buffer at pHs = 6, 7, 8 and 9. The excitation wavelength was 280 nm. The concentration of the enzyme was 0.17 mg/mL.

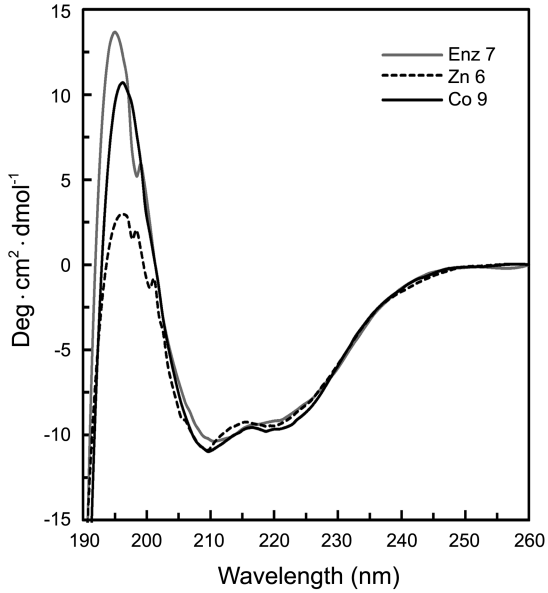


Figure 6. Far-UV CD spectra of MT : Sole enzyme in neutral pH (Enz7), with 0.2 mM Zn^{2+} at pH 6 (Zn6) and with 0.2 mM Co^{2+} at pH 9 (Co9).

other study, NaCl blocked proton (H^+) generation during L-DOPA oxidation.[40] Cl^{-1} reacts to the MT active site and binds to the enzyme-substrate complex (ES) state to form the ESI complex and shows a mixed inhibition manner.[29] Thus, as mentioned above the role of the substrate and the type of the ions are important for the enzyme activity. Co^{2+} is the ion most similar to Zn^{2+}. These two ions prefer tetrahedral rather than octahedral geometry at their chelates[41] Zn^{2+} has some characteristics in common with Cu^{2+} and both ions are good Lewis acid catalysts, but Zn^{2+} has no redox chemistry associated with it under biological conditions. Zn^{2+} is the metal ion cofactor in the active site of dopachrome tautomerase. To date we know of 12 zinc-enzymes whose Zn^{2+} ligands and modes of co-ordination have been identified; these represent examples of oxidoreductases, transferases, hydrolases and lyases.[42] Thus Zn^{2+} is preferable to almost all other cations as an acid-base catalyst in living systems on the grounds of availability, power and absence of redox properties.[42] Cobalt is rare in living systems, but it is a very valuable probe atom since its chemical similarity to zinc is such that it can replace it specifically,[43] as in carbonic anhydrase.[44] In this study, caffeic acid as a diphenol substrate play a crucial role in enzyme activation by Co^{2+} and Zn^{2+}. Jara *et al.*[25] in their studyed reported that Co^{2+} and Ni^{2+} increased tyrosinase hydroxylation in the melanogenesis pathway, while Zn^{2+} inhibited this activity in the mouse melanoma cells. In another study, Park *et al*.[28] showed that Zn^{2+} inhibits MT in a mixed type inhibition manner. They assessed the enzyme activity through proton generation from L-DOPA by thymol blue. In their thermodynamic analysis Zn^{2+} ligand-binding by isothermal titration calorimetry (ITC) showed that the enzyme was stabilized, and thus its hydrophobic surface was not exposed by a cation. Our results indicated that the maximum fluorescence intensity in the presence of Co^{2+} and Zn^{2+} decreased in comparison with the native enzyme spectra in the neutral pH. Hence, they induced enzyme stability and compactness. The imidazole ring is an essential metal binding site in metalloproteins and one or more imidazole units are bound to metal ions in almost all copper- and zinc metalloproteins, as seen in nickel-containing urease leading to profound effects on their biological actions.[45] The copper(II) is reported to promote the deprotonation of neutral imidazole ring, forming imidazolate bridged oligonuclear species, which could be observed only at pHs above 8 indicating that even a very weak basic imidazole ring may offer a particularly stable metal binding site.[46-48] The crucial role of copper in the active site of tyrosinase family and its replacement with other metals has been the focus of several previous studies. Based on the data obtained from photooxidation, active site-directed modification and amino acid sequence of *Neurospora* tyrosinase, it may be suggested that the histidyl residues188, 193, 289, and 306 represent possible ligands to the copper site in this enzyme.[49-51] Besides, it has been shown that Co^{2+} can replace Cu^{2+} in this enzyme, indicating that the histidines at the active site of the tyrosinase family enzymes can bind Co^{2+}.[52] In the present study, the Co^{2+} and Zn^{2+}-induced activation of MT obeyed the non-essential activation manner. They increased the maximum velocity of the enzyme due to the increase of the enzyme catalytic constant. Although the enzyme reached its optimum activity at pH 7, its optimum stability through fluorescence and CD structural analysis occurred at pH 8.

The overall data, including the metal affinity constants (K_a) in kinetic data and the assessment of the secondary and tertiary structures obtained by CD and fluorescence techniques, showed a considerable impact of Co^{2+} and Zn^{2+}

inducing structural stability of MT at pHs 9 and 6. Although the maximum activity of the sole enzyme was obtained at pH 7, its structure in the absence and presence of metal ions was found to be more fragile at this pH. Thus, the enzyme optimum activity failed to achieve its optimum structural stability conditions.

Acknowledgments. The financial support provided by the Research Councils of Tehran and Qazvin Universities of Medical Sciences is gratefully acknowledged. The authors also appreciate the sincere collaboration of Dr. A.A. Pahlevan for improving the linguistic quality of the text.

References

1. Solomon, E. I.; Sundaram, U. M.; Machonkin, T. E. *Chem. Rev.* **1996**, *96*, 2563.
2. Raper, H. S. *Physiol. Rev.* **1928**, *8*, 245.
3. Mason, H. S. *J. Biol. Chem.* **1948**, *172*, 83.
4. Rios, M.; Habecker, B.; Sasaoka, T.; Eisenhofer Rios, G.; Tian, H.; Landis, S.; Chikaraishi, D.; Roffler-Tarlov, S. J. *Neurosci.* **1999**, *19*, 3519.
5. Strack, D.; Schliemann, W. *Angew. Chem. Int. Ed.* **2001**, *40*, 3791.
6. Eisenhofer, G.; Tian, H.; Holmes, C.; Matsunaga, J.; Roffler-Tarlov, S.; Hearing, V. J. *FASEB J.* **2003**, *17*, 1248.
7. Lerch, K. *Life Chem. Rep.* **1987**, *5*, 221.
8. Seo, S.; Sharma, V. K.; Sharma, N. J. *Agric. Food. Chem.* **2003**, *51*, 2837.
9. Palumbo, A.; Misuraca, G.; D'Ischia, M.; Prota, G. *Biochem. J.* **1985**, *228*, 647.
10. Cicero, R.; Gallone, A.; Maida, I.; Pintucci, G. *Comp. Biochem. Physiol.* **1990**, *96*, 393.
11. Young, G.; Leone, C.; Strothkamp, K. G. *Biochemistry* **1990**, *29*, 9684.
12. Van Gelder, C. W. G.; Flurkey, W. H.; Wichers, H. J. *Phytochemistry* **1997**, *45*, 1309.
13. Gheibi, N.; Saboury, A. A.; Haghbeen, K. *Bull. Korean Chem. Soc.* **2006**, *27*, 642.
14. Gheibi, N.; Saboury, A. A.; Mansuri-Torshizi, H.; Haghbeen, K.; Moosavi-Movahedi, A. A. *J. Enz. Inhib. and Med. Chem.* **2005**, *20*, 393.
15. Gheibi, N.; Saboury, A. A.; Haghbeen, K.; Moosavi-Movahedi, A. A. *Colloids and Surfaces B: Biointerfaces* **2005**, *45*, 104.
16. Gheibi, N.; Saboury, A. A.; Haghbeen, K.; Moosavi-Movahedi, A. A. *J. Bioscience* **2006**, *31*(3), 355.
17. Gheibi, N.; Saboury, A. A. *J. Enz. Inhib. and Med. Chem.* **2005**, *24*, 1076.
18. Linder, M. C. In *Biochemistry of Copper*; Plenum: New York, 1991.
19. Lee, Y.; Won, H.; Lee, M.; Lee, B. *FEBS Letters* **2002**, *522*, 135.
20. Takahashi, K.; Akaishi, E.; Yumiko, A.; Ishikawa, R.; Tanaka, S.; Hosaka, K.; Kubohara, Y. *Biochem. Biophys. Res. Comm.* **2003**, *307*, 64.
21. Li, S.; Nakagawa, A.; Tsukihara, T. *Biochem. Biophys. Res. Comm.* **2003**, *324*, 529.
22. Buzadzic, B.; Korac, B.; Lazic, T.; Obradovic, D. *Food Res. Inter.* **2002**, *35*, 217.
23. Palumbo, A.; d'Ischia, M.; Misuraca, G.; Prota, G. *Biochim. Biophys. Acta* **1987**, *925*, 203.
24. Palumbo, A.; d'Ischia, M.; Misuraca, G.; Prota, G.; Schultz, T. M. *Biochim. Biophys. Acta* **1998**, *964*, 193.
25. Jara, J. R.; Solano, F.; Garcia-Borron, J. C.; Aroca, P.; Lozano, J. A. *Biochim. Biophys. Acta* **1990**, *1035*, 276.
26. Ancans, J.; Tobin, D. J.; Hoogduijn, M. J.; Smit, N. P.; Wakamatsu, K.; Thody, A. J. *Exp. Cell Res.* **2001**, *268*, 26.
27. Saboury, A. A. *J. Iran. Chem. Soc.* **2009**, *6*, 219.
28. Han, H. Y.; Zou, H. C.; Jeon, J. Y.; Wang, Y. J.; Xu, W. A.; Yang, J. M.; Park, Y. D. *Biochimica et Biophysica Acta* **2007**, *1774*, 822.
29. Park, Y. D.; Kim, S. Y.; Lyou, Y. J.; Lee, J. Y.; Yang, J. M. *Biochimie* **2005**, *87*, 931.
30. Beltramini, M.; Lerch, K. *Biochem. J.* **1982**, *205*, 173.
31. Longworth, J. W. In *Excited States of Proteins and Nucleic Acids*; Steiner, R. F., Weinryb, I., Eds.; Plenum Press: New York, 1971; 319.
32. Flesch, P. *Proc. Soc. Exp. Biol. Med.* **1949**, *70*, 79.
33. Bowness, J. M.; Morton, R. A. *Biochem. J.* **1953**, *53*, 620.
34. Bowness, J. M.; Morton, R. A.; Shakir, M. H.; Stubbs, A. L. *Biochem. J.* **1952**, *51*, 521.
35. Dorea, J. G.; Pereira, S. E. *J. Nut.* **1983**, *113*, 2375.
36. Molokhia, M. M.; Portnoy, B. *Br. J. Dermatol.* **1973**, *88*, 347.
37. Horcicko, J.; Borovansky, J.; Duchon, J.; Prochazkova, B.; Hoppe-Seyler's, Z. *Physiol. Chem.* **1983**, *354*, 203.
38. Tepper, A. W.; Bubacco, L.; Canters, G. W. *J. Biol. Chem.* **2002**, *277*, 30436.
39. Bubacco, L.; Salgado, J.; Tepper, A. W.; Vijgenboom, E.; Canters, G. W. *FEBS Lett.* **1999**, *442*, 215.
40. Park, Y. D.; Lee, J. R.; Park, K. H.; Hahn, H. S.; Hahn, M. J.; Yang, J. M. *J. Protein Chem.* **2003**, *22*, 473.
41. Solano, F.; Jimenez-Cervantes, C.; Martinez-Liarte, J. H.; Garcia-Borron, J. C.; Jara, J. R.; Lozano, J. A. *Biochem. J.* **1996**, *313*, 447.
42. Vallee, B. L.; Auld, D. S. *Biochemistry* **1990**, *29*, 5647.
43. Williams, R. P. *J. Polyhedron* **1987**, *6*, 61.
44. Lindskog, S.; Henderson, L. E.; Kannen, K. K.; Liljas, A.; Nyman, P. O.; Stranberg, B. *Enzymes* **1971**, *5*, 587.
45. Frausto da Silva J. J. R.; Williams, R. J. P. *The Biological Chemistry of the Elements; The Inorganic Chemistry of the Life*; Clarendon Press: Oxford, 1991.
46. Gajda, T.; Henry, B.; Delpuech, J. J. *J. Chem. Soc. Dalton Trans* **1992**, 2313.
47. Gajda, T.; Henry, B.; Delpuech, J. J. *J. Chem. Soc. Dalton Trans.* **1993**, 1301.
48. Gajda, T.; Henry, B.; Delpuech, J. J. *J. Inorg. Chem.* **1995**, *34*, 2455.
49. Pfiffner, E.; Dietler, C.; Lerch, K. In *Comparative Study and Recent Knowledge on Quaternary Structure and Active Sites of Oxygen Carriers and Related Proteins*; Tours, France, Marcel Dekker: New York, 1980.
50. Dietler, C.; Lerch, K. In *Proceedings ofthe Third International Symposium on Oxidases and Related Redox Systems*; Mason, H. S., King, T., Morrison, M., Eds.; Academic Press: New York 1979.
51. Lerch, K. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3635.
52. Ruegg, C.; Lerch, K. *Biochemistry* **1981**, *20*, 1256.